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(54) Title: DETECTING OR QUANTIFYING MULTIPLE ANALYTES USING LABELLING TECHNIQUES

(57) Abstract

A method for the assay, detection etc. of each of a plurality of substances of interest in a sample comprises labelling each of the substances with one or more components each capable of taking part in a respective distinguishable chemiluminescent reaction. Luminescent reagents for use in the method are also disclosed.

* See back of page

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"Detecting or Quantifying Multiple Analytes using labelling techniques"

This invention relates to methods and reagents for the assay, detection, quantification, location or analysis of each of a plurality of substances of interest ("analytes") in a sample in which each substance is linked ("labelled") with another molecule or molecules capable of taking part in a chemiluminescent reaction.

For the purposes of this specification, a chemiluminescent reaction is defined as one which involves a chemical reaction that results in the emission of electromagnetic radiation. This luminescence is to be distinguished clearly from fluorescence and phosphorescence. Here, luminescence, or more precisely, chemiluminescence also encompasses light emission from biological reactions (bioluminescent reactions).

A luminescent reaction is normally one between at least two molecules (S and L) with or without other reagents, cofactors, or a catalyst (D) or under the influence of a physical trigger. L is the substance which generates light, such as luminol. S is the substance which reacts with L to cause excitation, for example oxygen or hydrogen peroxide. D (if present) is a cofactor, and/or catalyst or trigger such as an enzyme, a luciferase, or potassium ferricyanide. The reaction between L and S results in the conversion of L

to an excited molecule L* and the return of this excited molecule to a non-excited state results in the emission of a photon. The reaction between L and S and the decay of L* to the non-excited state may take place spontaneously or may require the presence of the cofactor or catalyst D, or a physical trigger such as temperature. An example of such a reaction is the oxidation by H O of luminol. The catalyst and cofactors are often inorganic compounds as here, but may also be extracted from biological material such as the enzyme peroxidase which catalyses the luminescent reaction involving luminol.

These methods and reagents discussed above may be used in a wide variety of techniques such as immuno-assays, protein binding assays, nucleic acid hybridisation assays, cellular receptor binding assays and other analogous techniques which involve binding of the substance of interest with a specific binding partner or reagent. These types of linking are referred to herein as "binding or otherwise linking with".

The substances of interest may be peptides, proteins, polypeptides, nucleic acids and other substances of biological interest.

Binding assays have been used for many years in the quantitation of molecules of biological interest. Numerous examples have been described in which the binding step is an immunological reaction, a protein binding reactiion, reaction with a cellular receptor or a complementary nucleic acid hybridisation reaction. Sensitive assays based on

these reactions require the use of a label which can be attached or incorporated into one of the binding partners of such a reaction such that the degree of binding and hence the concentration or mass of another component of the reaction - the substance of interest - can be determined. Many variations of the basic binding reactions have been described and many different labels used, including radio-isotopes, enzymes, fluorescent molecules and chemi-luminescent molecules.

Various combinations of these have been used in sequence for the detection and quantitation of a wide variety of analytes ranging from small molecules such as hormones and drugs to large molecules such as nucleotides.

Generally speaking, these techniques have only been applied to the investigation of a single analyte in one test reaction, but there have been a limited number of examples where two analytes have been determined essentially using a single test procedure. The best known of these have been simultaneous immunoassays and/or protein binding assays for vitamin B12 and folic acid and also for thyroxine and thyro-trophin. In these cases the two different reactions are monitored independently using a different radioactive isotope for each. Here use is made of cobalt-57 and iodine-125 whose radioactive emissions are distinguishable using an appropriate gamma counter. Similar strategies have also been used for the simultaneous determination of lutrophin and follitrophin.

Radioactive reagents have three major disadvantages.

Firstly, the method of labelling involves the use of highly radioactive and hence potentially hazardous reagents. Secondly, the shelf life of the radioactively labelled substance is often relatively short not only because by its very nature the radioactive isotope is continuously decaying, but also radioactively labelled proteins are often unstable. Thirdly, it is often difficult to label proteins sufficiently to provide a sensitively and rapidly detectable reagent. The measurement of luminesence is both highly sensitive and very rapid, the time of measurement being of the order of seconds rather than the several minutes normally required for measurement of radioactivity. The attachment either covalently or non-covalently, to substances not normally capable of taking part in a luminescent reaction of a substance which is capable of taking part in a luminescent reaction provides a reagent which can be rapidly measured in very small quantities.

Work has been described relating to the use of different fluorescent molecules in so-called "dual labelling" systems. However, fluorescent labelling systems are usually capable of only gross analysis of substances and are not generally suitable for sensitive analysis. Also, with fluroescent systems, the sample is illuminated by U.V. radiation to measure the fluoresence and this may cause major problems due to photobleaching. Multiple analyte immunoassays based on the use of fluorophores have been described in which the different labels used have been chelates of different lanthanide metals emitting at

different wavelengths. Limitations arise here because certain of the fluorophores used have low quantum yields and generally all assays based on these materials require complex instrumentation and chemistries in order to achieve the high levels of performance which are characteristic of many chemiluminescent systems (Ref.1).

Broadly stated, according to one aspect of this invention, there is provided a method for the assay, detection, quantification, location or analysis of each of a plurality of substances of interest contained in a sample, which comprises labelling each of said substances with one or more components capable of taking part in a respective distinguishable chemiluminescent reaction.

In another aspect, this invention provides a method for the assay, detection, quantification, location of analysis of a sample containing at least two substances of interest which comprises:-

(i) treating said sample to form at least first and second complexes, said first complexes being made up of one of said substances, or a respective associated substance, bound or otherwise linked with a first reagent capable of taking part in a first chemi-luminescent reaction and said second complexes being made up of another of said substances, or a respective associated substance, bound or otherwise linked with a second reagent capable of taking part in a second chemiluminescent reaction which has emission characteristics distinguishable from those of said

first chemiluminescent reaction;

(ii) subsequently treating said sample containing said first and second complexes to cause said first and second chemiluminescent reactions to occur, and

(iii) observing, sensing, measuring and/or recording the emission of each of said chemiluminescent reac tions.

In yet another aspect, this invention provides a luminescent reagent which comprises a mixture of at least two substances capable of binding or otherwise linking with respective different binding partners, one of said substances being labelled with one or more components capable of taking part in a respective one chemiluminescent reaction and another of said substances being labelled with one or more components capable of taking part in a respective other chemiluminescent reaction of which the emission characteristics are distinguishable from said one chemiluminescent reaction.

We have found that different chemiluminescent labels can be produced which, by appropriate chemical manipulation, possess different characteristics in terms of the speed and wavelength of light emission and that these different labels can be used advantageously in analyte binding systems to permit the substantially simultaneous quantitation of two or more different analytes within a single test procedure.

It is known that certain changes in the structure of chemiluminescent molecules cause changes in the spectrum of the emitted light. Recently it has been suggested that conventional enzyme immunoassays with light emitting end

points could be used for multiple assays (Ref.2) though it is not taught how this may be achieved nor is it obvious how such assays would be performed. Neither are there any data to support this suggestion. No comparable suggestions have been made for situations in which luminescent molecules themselves are linked to substances of biological interest as opposed to using luminescent end-point enzyme immuno-assays. The advantages of using such direct labelling as opposed to enzymic modulation are well established and yield advantages in terms of simplicity, robustness and sensitivity. No teachings exist as to how the advantages of such systems may be exploited for multiple analyte assays nor is it obvious what instrumentation would be required etc. in order to perform such assays. More recently it has been shown that modification of the luciferase component of bioluminescent reactions by genetic manipulation (Ref.3) forms an additional means of modifying the wavelength of emission from such reactions as an alternative to modifying the chemical structure of the corresponding luciferin.

In a further aspect it is known that, generally, change in chemical reaction conditions or structural changes within the reactant molecules themselves may affect the rates of their various possible reactions. Similarly, more specifically, the kinetics of chemiluminescent reactions are often affected by the chemiluminescent molecule. It has been suggested for example (Ref.4,5) that the rates of chemiluminescent reactions of acridinium salts are dependent on certain structural features. Likewise it has been

reported that enzyme driven chemiluminescent reactions may be affected by the structure of the substates (Ref.6). That these kinetic effects may be utilised in the development of multiple assays based on such kinetic differences has not been taught, neither is it obvious from any related art how such assays may be configured and utilised.

A discussion of non-limiting embodiments of the invention now follow, together with a specific example of an assay procedure, reference being made to the accompanying drawings, in which:-

Figure 1 is a schematic graph showing emission intensity vs. time a typical test procedure according to the invention; and

Figure 2 is a schematic graph showing emission intensity vs. time in an example of procedure according to the invention for the immunochemiluminometric assay for human gonadotrophin and human alpha-fetoprotein.

General Scheme

A and B are the analytes of interest and are each capable of binding more than one antibody molecule so that parallel two-site immunoassays can be set up. A mixture of antibodies capable of binding A and B is coated on to the walls of a test tube. The sample for analysis containing unknown amounts of A and B is added to the tube together with a mixture of soluble complementary antibodies capable of binding to other sites on A and B. The soluble antibodies specific for A and B are labelled with chemiluminescent molecules exhibiting distinguishable charac-

teristics, e.g. fast and slow light emission. Following an appropriate incubation period, two-site immune complexes will be formed on the sides of the tube, the extent of immune complex formation depending on the amount of A and B present. Following removal of unbound substances by aspiration of the soluble contents of the tube, the chemiluminescence emission remaining is triggered and then measured in a luminometer. The total number of photons emitted is proportional to the total amount of A and B present. However, in this example, the chemiluminescence emission from labelled antibodies specific for A is rapid and complete within one second whereas the emission from labelled antibodies specific for B is much slower and reaches a peak after initiation before decaying over the next nineteen seconds (see Figure 1). Thus measurement of the photons emitted in two separate time windows of 1 and 19 seconds within an overall measuring time of 20 seconds permits independent quantitation of A and B upon calibration of the system.

Assay Techniques and Analytes

The methods in accordance with the invention may quantify those species of biological interest which are identifiable using single analyte quantitation techniques. The following examples are given with guidance as to the type of binding reaction used. This list is given for example only and does not imply any limitations of the invention:-

1. Immunoassay:

   Drugs, vitamins, steroids, thyroid hormones, peptides, polypeptides, proteins, immunoglobulins, viruses, bacteria, protozoa.

2. Protein binding assays:

   Vitamins, cofactors, enzyme inhibitors.

3. Nucleic acid hybridisation assays:

   Oligonucleotides, polynucleotides, DNA, RNA, onocogenes, microorganisms.

4. Receptor binding assays:

   Progestogen receptors, estrogen receptors, thyrotrophin receptors, thyroid hormone receptors.

Certain pairs of groups of analytes are often measured to get a more complete picture of the biological system or to improve efficiency of testing of the biological system. In such cases the availability of simultaneous, multi-analyte measurement offered by the invention is uniquely advantageous. Examples of such groups of analytes are given below but do not imply limitations of the invention.

1. Hormones:

   Thyroxin/thyrotrophin, lutrophin/follitrophin, adrenocorticortrophin/cortisol.

2. Vitamins and cofactors:

   Vitamin B12/folic acid, 1,25-dihydroxycholical-ciferol/25-hydroxycholecalciferol.

3. Nucleic acids (from viruses and micro-organisms):

   Neisseria Gonorrhoea/Chlamydia Trachomatis.

4. Tumour markers:

Prostate Specific Antigen/Prostatic Acid Phosphatase, alphafetoprotein/carcinoembryonic antigen/chorionic gonadotrophin.

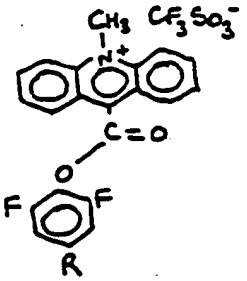
Labels

The preferred way of associating chemiluminesence activity with the appropriate binding reaction is to chemically or physically couple a component such as a chemiluminescent molecule, capable of taking part in a chemiluminescent reaction, to one of the components of that binding reaction so as to produce a specific labelled reagent. The luminescent reagents according to the present invention will thus include two or more such labelled reagents each carrying a label having different characteristics in terms of kinetic and/or spectroscopic properties. Each of these labelled reagents will have a particular specificity for taking part in a given binding reaction, thus each given binding reaction can be monitored independently even though two or more such reactions are occurring simultaneously. Hence it is possible to quantify, independently and simultaneously, the analytes taking part in these parallel binding reactions.

Different members of a number of classes of chemiluminescent molecules are capable of exhibiting differences in kinetic and/or spectroscopic properties and can hence be used in the invention, including acridinium and related compounds (e.g. phenanthridinium compounds), phthalhydrazides and related compounds (e.g. naphthalhydrazides),

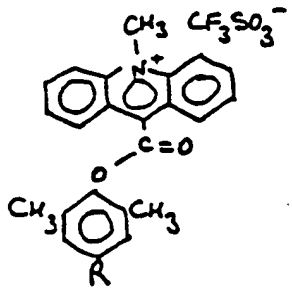
oxalate esters and related compounds and also stabilised dioxetanes and dioxetanones. The variations of compounds within such groups are well-known to those moderately skilled in the art, likewise it is known that the quantum yield, kinetics and emission wavelengths of their chemiluminescent reactions are affected by their structure (see earlier and also Refs. 7-11). Thus the structures of a plurality of compounds differing in kinetics or emission wavelength parameters, suitable for use in this invention are individually readily conceived by one skilled in the art. For example, from the existing literature, one skilled in the art would know to choose from those compounds with high quantum yields, and to choose those compounds which, relative to each other possessed substantial differences in their reaction rates or their emission wavelengths, in order to maximise the resolution between the detection of these compounds. Aryl acridinium esters may be used as labels with appropriate chemical modifications made to produce the desired kinetic and spectroscopic parameters. Some examples of such compounds are given below and do not imply any limitations of the invention.

1. Kinetic variation

a. 

Duration of light emission under standard conditions = 0.8 seconds

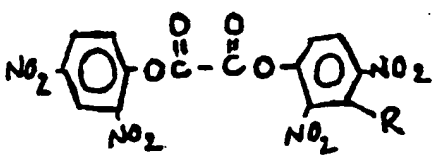
b.



Duration of light emission under standard conditions = 60 seconds

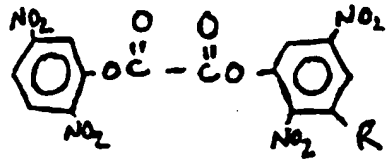
In the acridinium phenyl ester of 1a. the phenyl moiety is substituted with F groups which are electron withdrawing and thus modify the acridinium phenyl ester so that the emission of light occurs over a relatively short period. In the acridinium phenyl ester of 1b, the phenyl moiety is substituted with CH_3 groups which are electron donating so that the emission of light occurs over a relatively long period. Other electron donating and withdrawing groups may be used.

c.



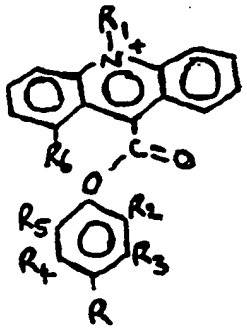
Duration of light emission under standard conditions (9,10-diphenylanthracene fluorescent acceptor) = 30 min

d.



Duration of light emission under standard conditions (9,10 - diphenylanthracene fluorescent = 2 min

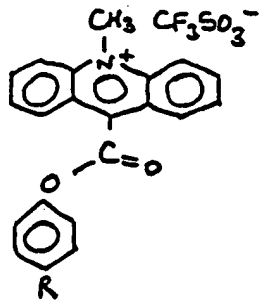
Table 1 gives further examples for the series



	R_1	R_2	R_3	R_4	R_5	R_6	$t_{½}$(decayphase half-life)
	CH_3	H	H	H	H	H	0.6s
	CH_3	CH_3O	H	H	CH_3O	H	7s
	$C_6H_5CH_2$	H	H	H	H	H	0.5s
	CH_3	CH_3	H	H	H	H	11s
	CH_3	NO_2	H	H	NO_2	H	< 0.4s
	CH_3	H	CH_3	CH_3	H	H	0.7s
	CH_3	CH_3	H	H	CH_3	CH_3	4s
	CH_3	Br	H	H	Br	H	< 0.4s

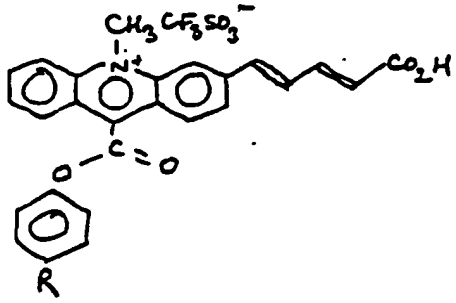
2. Spectroscopic variation

a.



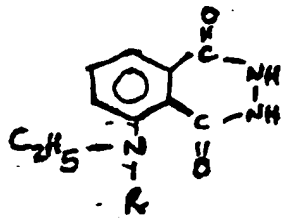
Emission λ max ~ 430 nm

b.



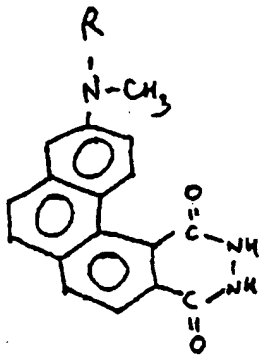
Emission λ max ~510 nm

c.



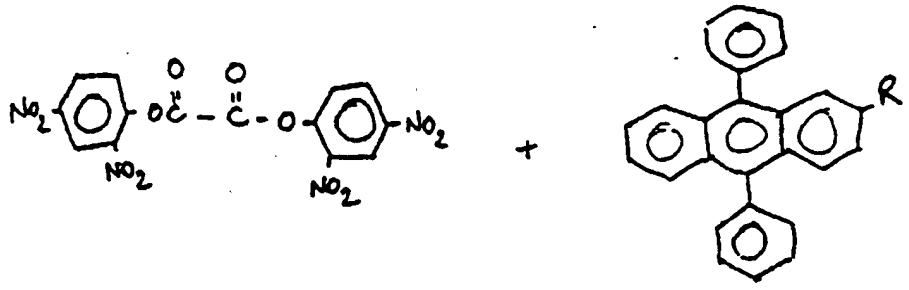
Emission

λmax ~ 430 nm

d.  λmax - 520 mm

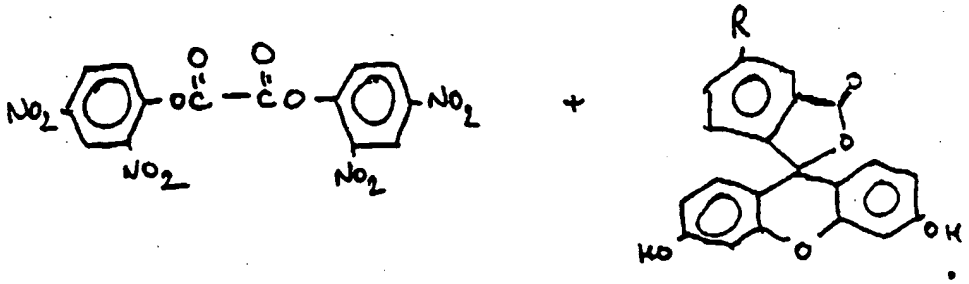
e.

λmax - 430 mm



f.

λmax - 512 mm



In 2b, the electronic conjugation of the nucleus has been increased so that the emission radiation is of relatively long wavelength.

In each of the acridinium compounds illustrated above, R is selected to allow covalent coupling to a component of the appropriate binding reaction. Appropriate coupling groups are well described but in this example are selected such that the desired kinetic and/or spectroscopic properties of the molecule are maintained and also that the final labelled reagent is still active in terms of its ability to participate in the binding reaction. Such groups include N-hydroxysuccinimide esters, imidate esters, isothiocyanates and other established active group or groups that can give rise to active groups to facilitate coupling to molecules of biological interest. Preferably these groups are linked to the chemiluminescent moiety by an aliphatic chain of appropriate length.

In a further aspect it is possible to also make use of chemiluminescent reactions which involve energy transfer to a fluorescent acceptor molecule. As an example of this it is possible to label an antibody of one specificity with fluorescein and an antibody of another specificity with rhodamine. These antibodies can be used in simultaneous two-site assays and the end-points determined by introduction of a peroxyoxalate chemiluminescence system (hydrogen peroxide/bis-2, 4-dinitrophenyl oxalate). Radiationless energy transfer occurs resulting in the emission of light at two different wavelengths (green-yellow from fluorescein/red from rhodamine), the intensities of the emissions are directly proportional to th amount of the relevant labelled

antibody bound in the immunochemical reactions.

Analyte-binding partners (reagents) and binding reactions

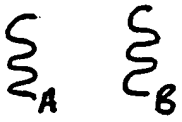
The following schemes represent examples of binding partners or binding reactions that it is possible to use for the determination of concentrations of single analytes which are used currently:

Key:

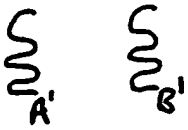
Solid phase matrix (e.g. coated tube, magnetisable particles)

Different analytes represented by A and B

Antibodies specific for A and B respectively or binding proteins specific for A and B respectively or receptors capable of binding A and B respectively



Oligonucleotide sequences A and B

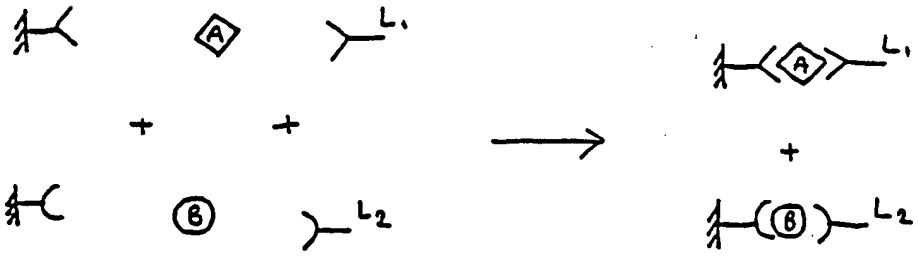


Complementary oligonucleotide sequences

Binding reagent for double-stranded (recombinant) nucleic acid sequences

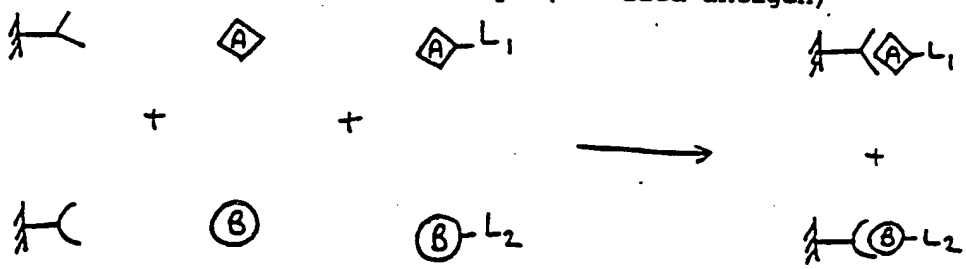
Chemiluminescent labels exhibiting different r action kinetics and/or spectroscopic properties respectively.

1a. Two-Site immunoassays.

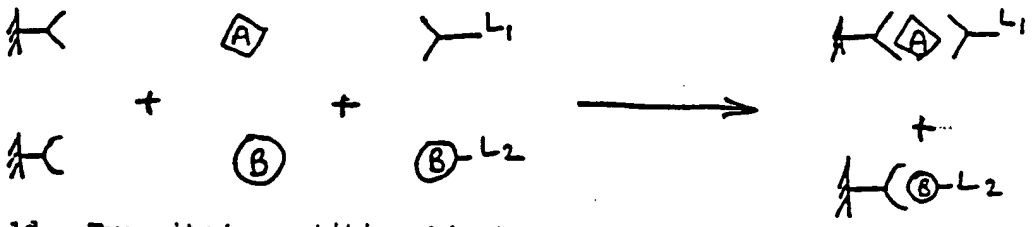


Antibodies of a given specificity for the analyte recognise different parts of the given analyte to permit formation of the two-site immune complex. Excess concentrations of reagent over analyte are used in two-site systems.

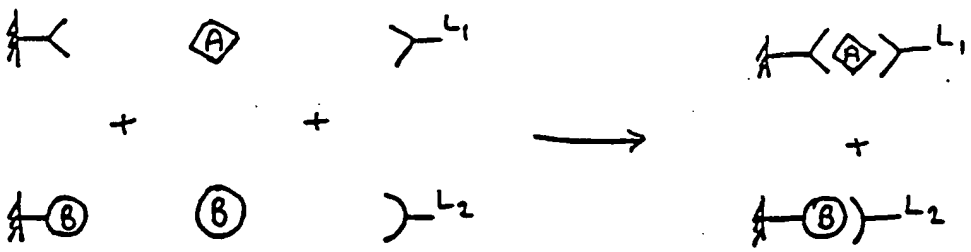
1b Competitive binding immunoassays (labelled antigen)



1c Two-site/competitive binding (labelled antigen) combination.

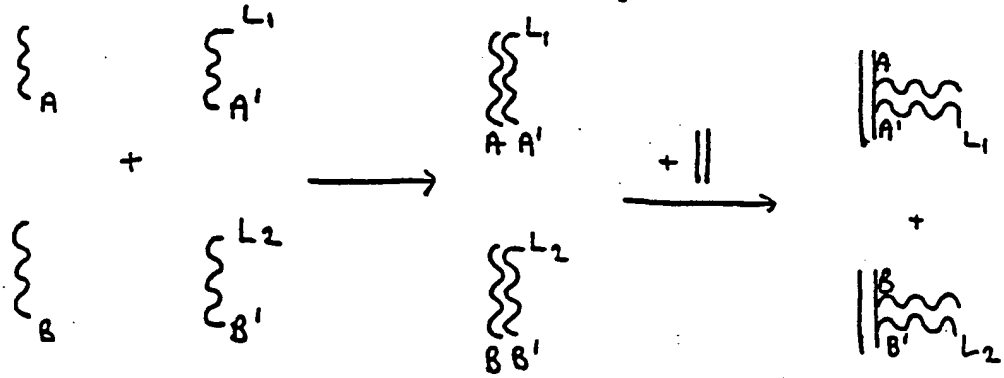


1d Two-site/competitive binding (labelled antibody) combination.



It should be noted that limiting reagent concentrations are used in competitive binding systems.

2. Oligonucleotide hybridisation assays



The above are all examples of heterogeneous assay systems in which the analyte/binding-partner complex is isolated from uncomplexed material. Additionally it is possible to apply the disclosed systems to homogeneous assays.

Instrumentation

Photon counting equipment may be used for the measurement of light intensity. The sensing equipment should be capable of distinguishing th· emissions from the distinguishable chemiluminescent reactions.

1 A Kinetic discrimination.

As described in the earlier example, the equipment should be capable of recording measurements of light

intensity (preferably as photon counts per unit time) within at least two time frames to permit independent measurement of the intensity arising from slow and fast reactions. In many instances there will be overlap between the two signals which is accounted for by appropriate selection of time frames or by mathematical estimation of the overlap.

2. Spectroscopic discrimination:

Here it is necessary to measure the intensity of two or more wavelengths simultaneously. This can be achieved, e.g. by use of the necessary number of photomultiplier tubes each fitted with a bandpass interference filter to permit measurement of one signal at the exclusion of others. Alternatively a single photomultiplier tube can be used such that the light emitted from the reaction is first passed through a fast scanning spectrometer or filter/chopper system. Synchronisation of the photomultiplier tube output with the scanning or chopping frequency thus permits independent quantitation of the different wavelengths.

SPECIFIC EXAMPLE

1. Preparation of labelled antibodies:

a. 4-(2-carbonylethyl) phenyl-10-methyl acridinium-9-carboxylate fluorosulphonate labelled antibodies to human chorionic gonadotrophin (hCG).

The acridinium label was synthesised as follows: acridinium-9-carboxylic acid (5g) was refluxed with thionyl chloride (15 ml) for 3 h urs. The solvent was removed under reduced pressure and the product suspended in anhydrous

pyridine (35 ml). Benzyl 4-hydroxyphenylpropanoate (9 nmol) was added and the solution stirred overnight at room temperature. The mixture was subsequently poured into crushed ice/1 M hydrochloric acid (250 ml) and the resulting precipitate filtered, washed with water and dried under reduced pressure. The 4-(2-benzyloxycarbonylethyl)phenyl-9-acridine carboxylate thus obtained was recrystallised from benzene/cyclohexane. 0.46 g of this was dissolved in hydrogen bromide/acetic acid mixture (45/55 w/w, 10 ml) and the solution stirred for 2 h at 50-55°C. The solution was poured into water (100 ml) and the resulting yellow solid, filtered, washed with water and dried under reduced pressure, thence recrystallised from acetonitrile/chloroform to yield 4-(2-carboxyethyl)phenyl-9-acridine carboxylate. N-hydroxysuccinimide (62 mg) was dissolved in dimethyl-formamide (5 ml) together with 200 mg of the above acridine carboxylate. The mixture was cooled to -20°C and dicyclohexylcarbodiimide (123 mg) added, followed by stirring for 2 h at -20°C, thence overnight at room temperature. One drop of glacial acetic acid was then added and the mixture left for a further 30 min. The dicyclohexylurea was removed by filtration and the material obtained by evaporation of the liquor was recrystallised from benzene/cyclohexane to yield 4-(2-succinimidyloxy carbonylethyl)phenyl-9-acridine carboxylate. The product (234 mg) was dissolved in anhydrous chloroform (25 ml) and methyl fluorosulphonate (0.5 ml) added.The precipitate which formed after stirring at room temperature for 18 hours was

filtered and washed with anhydrous benzene to yield 4-(2-succinimidyloxycarbonylethyl)phenyl-10-methylacridinium-9-carboxylate fluorosulphonate. Mouse monoclonal antibodies (50 µg) raised to human chorionic gonadotrophin were dissolved in sodium phosphate buffer (pH 7.4, 0.1 M, 200 µl) containing 0.15 M sodium chloride. A stock solution was made of the acridinium-succinimidyl ester in acetonitrile (0.5 mg/ml) and 10 µl added to the antibody solution with mixing. After incubation at room temperature for 15 min in the dark, a solution of lysine monohydrochloride (100 µl, 10mg/ml) in the above buffer was added and the mixture left for a further 5 min. The mixture was purified on a column of Pharmacia Sephadex G25-M (30 cm x 0.6 cm) equilibrated and eluted with phosphate buffered saline (pH 6.3, 0.1 M, 0.15 M NaCl) containing 0.1% (w/v) bovine serum albumin and 0,05% (w/v) sodium azide. 0.5 ml fractions were collected and the void volume fractions pooled and stored at 4°C.

b. 4-(2-imidylethyl)-2,6-dimethyl-10-methylacridinium-9-carboxylate dichloride labelled antibodies to human alpha-fetoprotein (AFP).

The acridinium label was synthesised as follows: acridinium-9-carboxylic acid (2.5 g) was refluxed with thionyl chloride (10 ml) for 3 hours. The solvent was removed under reduced pressure and the product suspended in anhydrous pyridine (25 ml). 2,6-dimethyl-4-hydroxy-phenylpropionitrile (1.3 g) was added and the solution stirred overnight at room temperature. The mixture was subsequently poured into crushed ice/1 M hydrochloric acid

(250 ml) and the resulting precipitate filtered, washed with water and dried under reduced pressure. The 4-(2-cyanoethyl)-2,6-dimethylphenylacridinium carboxylate thus obtained was dissolved in anhydrous chloroform (15 ml) and methyltrifluoromethylsulphonate (0.5 ml) added. The precipitate which formed after stirring overnight at room temperature and addition of diethylether was filtered off and washed with anhydrous benzene to yield 4-(2-cyanoethyl)-2,6-dimethyl-phenyl-10-methyl-9-acridiniumcarboxylate trifluoromethylsulphonate which was subsequently dissolved (69 mg, 10 ml) in anhydrous methanol. HCl gas was bubbled through the solution under nitrogen, kept at ice temperature for 2 hours and left to stand for a further 1 hour. The crystals formed were filtered under an atmosphere of dry nitrogen and washed with anhydrous methanol to yield 4-(2-methyloxyimidylethyl)-2,6-dimethylphenyl-10-methylacridinium-9-carboxylate dichloride. Mouse monoclonal antibodies (50 µg) raised to human alpha-fetoprotein were dissolved in sodium borate buffer (pH 9.5, 0.1 M, 200 µl) containing 0.15 M sodium chloride. A stock solution was made of acridinium imidoester in acetonitrile (0.5 mg/ml) and 190 µl added to the antibody solution with mixing. After incubation at room temperature for 30 min in the dark, a solution of lysine monohydrochloride (100 µl, 10mg/ml) in the above buffer was added and the mixture left for a further 15 mins. The mixture was purified as described above.

2. Preparati n of solid-phase antibodies

M noclonal antibodies to human chorionic g nadotr -

phin and alpha-fetoprotein recognising distinct epitopes to those recognised by the labelled antibodies were coupled to paramagnetic particles using published methods.

3. "Simultaneous" immunochemiluminometric assay for human chorionic gonadotrophin and human alpha-fetoprotein.

Solid phase antibody suspensions (800 µg/ml) were mixed in equal volumes. Labelled antibody solutions (10 ng/ml anti-hCG, 50 ng/ml anti-AFP) were mixed in equal volumes. Diluent buffer was same as antibody purification buffer (above). Standard mixtures for calibration consisted of solutions containing known concentrations of hCG and AFP in horse serum. 50 µl of patient serum sample were dispensed in duplicate into 12 x 75 mm polystyrene test tube. Standard tubes were set up in duplicate using 50 µl of the appropriate standard. 100 µl of the labelled antibody mixture were added followed by 100 µl of the solid-phase antibody mixture. The tubes were mixed and set aside for 1 hour at room temperature. 1ml of wash solution (1.76 g/l sodium dihydrogen orthophosphate, 0.15M sodium chloride, 0.5% (W/v) sodiumaxide, 0.5% bovine serum albumin, 1% (v/v) Triton X-100) was added and the tubes placed in a magnetic rack to facilitate sedimentation of the solid phase. The supernatants were decanted to waste and a further 1 ml of wash buffer added followed by mixing of the tube contents. A further sedimentation/decantation step was performed and the tubes placed in a luminometer.

4. Measurement of light intensity.

Measurements of light emission were made in a Ciba

Corning Magic Lite Analyzer using procedures recommended by the Manufacturer. Manipulation of the software enabled distinct, sequential integration of light intensity with respect to time. Separate integrations were performed in the ranges 0 - 1 and 1 - 2 seconds corresponding to light emission from the hCG and AFP antibodies respectively and hence being proportional to the concentrations of hCG and AFP in the sample. Since some overlap existed due to interference with the hCG signal by the AFP signal, correction was necessary. This was achieved by estimating the AFP contribution to the 0 - 1 second integration using the previously determined relationship of the light intensity at 2 seconds to the integral between 0 - 1 seconds, as can be seen in Figure 2.

[hCG] U/L	Photon Counts	[AFP] kU/L	Photon Counts	Overlap	Corrected hCG Photon Counts
250	1.22×10^6	250	1.14×10^5	3.35×10^4	1.19×10^6
125	8.11×10^5	125	7.4×10^4	2.18×10^4	7.89×10^5
62.5	4.66×10^5	62.5	4.2×10^4	1.24×10^4	4.54×10^5
31.3	2.63×10^5	31.3	2.3×10^4	6.76×10^3	2.56×10^5
15.6	1.33×10^5	15.6	1.3×10^4	3.82×10^3	1.29×10^5
0	3.37×10^3	0	6.6×10^2	1.94×10^2	3.18×10^3

r=3.40

When [hCG] >> [AFP] the residual hCG signal during the

second time window made a significant contribution hence perturbing measurement of x. This was not a problem in practice and could be minimised by relative assay optimisation e.g. by manipulation of specific activities of the labelled antibodies.

In situations where accuracy is required even at extreme relative concentrations mutual overlap is accounted for by the use of more complex software capable of iterative or simulatenous equation calculation.

Where overlap is a serious problem alternative labels must be sought that exhibit greater differences in their relative kinetic properties.

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11. U.S. Patent No. 3817837 (Rubenstein)

The subject matter listed above is incorporated herein by reference

CLAIMS

1. A method for the assay, detection, quantification, location or analysis of each of a plurality of substances of interest contained in a sample, which comprises labelling each of said substances with one or more components capable of taking part in a respective distinguishable chemiluminescent reaction.

2. A method for the assay, detection, quantification, location or analysis of a sample containing at least two substances of interest which comprises treating said sample to label one of said at least two substances, or a respective associated substance, with one or more components which can take part in a first chemiluminescent reaction, and to label another of said at least two substances, or a respective associated substance, with one or more components which can take part in a second chemiluminescent reaction, wherein the emission characteristics of said first and second chemi-luminescent reactions are distinguishable from each other,

subsequently causing said chemiluminescent reactions to occur, and observing, sensing, measuring and/or recording the emissions of each of said chemiluminescent reactions.

3. A method for the assay, detection, quantification, location or analysis of a sample containing at least two substances of interest which comprises:

(i) treating said sample to form at least first and second complexes, said first complexes

being made up of one of said substances, or a respective associated substance, bound or otherwise linked with a first reagent capable of taking part in a first chemiluminescent reaction and said second complexes being made up of another of said substances, or a respective associated substance, bound or otherwise linked with a second reagent capable of taking part in a second chemiluminescent reaction which has emission characteristics distinguishable from those of said first chemiluminescent reaction;

(ii) subsequently treating said sample containing said first and second complexes to cause said first and second chemiluminescent reactions to occur, and

(iii) observing, sensing, measuring and/or recording the emissions of each of said chemiluminescent reactions.

4. A method according to Claim 3, wherein said step of treating to form first and second complexes includes reacting said sample with a mixture containing said first and second reagents.

5. A method according to Claim 3 or Claim 4, wherein said first and second chemiluminescent reactions are caused to occur by effecting substantially simultaneous triggering of said chemiluminescent reactions.

6. A method according to any preceding claim, wherein the emission characteristics of each of said chemiluminescent reactions are distinguishable in terms of the variation of light emission or radiatiion intensity with time of the emissions.

7. A method according to Claim 6, wherein the intensity of the radiation emitted from said sample is observed, sensed, measured and/or recorded over two different time intervals which may overlap.

8. A method according to any of Claims 1 to 5, wherein each of said chemiluminescent reactions emits radiation in a respective different specrtral range.

9. A method according to any of Claims 1 to 5, which involves at least three chemiluminescent reactions, of which at least two are distinguishable in terms of the spectral emission characteristics and at least two are distinguishable in terms of the variation of light or radiation intensity with time.

10. A method according to Claim 8 or 9, wherein the emissions are filtered by respective filtering means responsive to radiation in said different spectral ranges and the filtered intensities of the emissions are observed, sensed, measured and/or recorded.

11. A method according to Claim 3 or any claim dependent thereon, wherein at least one of said first and second complexes is made up by a reagent bound to a substance by one of an immunoassay binding reaction, a protein binding reacti n, a nucleic acid hybridisation and a

receptor binding reaction.

12. A method according to any preceding claim, wherein each of said chemiluminescent reactions includes a respective component or reagent which is a structural variant of the or each component or reagent associated with the or each other chemiluminescent reactions.

13. A method according to any of the preceding claims, wherein one of the components in at least one of said chemi-luminescent reactions is based on acridinium compounds, or structural variants thereof.

14. A method according to Claim 13, wherein one of the components in at least one of said chemiluminescent reactions is an acridinium salt alkylated at the ring nitrogen.

15. A method according to Claim 124, wherein one of said components in at least one of said chemiluminescent reactions is an acridinium phenyl ester wherein the ester is formed at position 9 of the acridine nucleus.

16. A method according to Claim 15, wherein one of said chemiluminescent reactions includes an acridinium phenyl ester wherein the phenyl moiety is substituted with electron withdrawing groups to yield a chemiluminescent reaction in which the emission of light occurs over a relatively short period, and another of said chemiluminescent reactions includes an acridinium phenyl ester wherein the phenyl moiety is substituted with electron donating groups to yield a chemiluminescent reaction in which the emission of light occurs over a relatively long period.

17. A method according to Claim 14, wherein one of said chemiluminescent reactions includes an acridinium salt which, on triggering a said reaction, emits radiation at a relatively short wavelength and wherein another of said chemiluminescent reactions includes an acridinium salt wherein the electronic conjugation of the acridine necleus is increased whereby, on triggering of said another reaction, said acridinium salt emits at a relatively long wavelength.

18. A method according to Claim 17, wherein said one chemiluminescent reaction emits radiation of wavelength in the range of from 400 to 500 nm and said other chemiluminescent reaction emits radiation of wavelength in the range of from 500 to 700 nm.

19. A method according to Claim 13 or any claim dependent thereon wherein the acridinium compound is modified or further derivatised to permit covalent coupling to a molecule of biological interest.

20. A method according to any of Claims 1 to 12, wherein one of the components in at least one of said chemiluminescent reactions is based on one of the following compounds:

(i) phthalhydrazides and related compounds

(ii) dioxetanes, dioxetanones and related compounds

(iii) Bis-oxalate esters, related compounds and associated acceptor partners where required.

21. A method for the assay, detection, quantification or location of each of a plurality of different substances of interest contained in a sample, wherein the sample is reacted with a mixture of appropriate binding partners for binding or otherwise linking with respective ones of said different substances, said binding partners each being associated with a respective chemiluminescent labelling system which is distinguishable from the chemiluminescent labelling systems associated with the other binding partners.

22. A luminescent reagent which comprises a mixture of at least two substances capable of binding or otherwise linking with respective different binding partners, one of said substances being labelled with one or more components capable of taking part in a respective one chemiluminescent reaction and another of said substances being labelled with one or more components capable of taking part in a respective another chemiluminescent reaction of which the emission characteristics are distinguishable from said one chemiluminescent reaction.

23. A luminescent reagent according to Claim 22, wherein each of said chemiluminescent reactions are distinguishable from each other in terms of the variation of light intensity with time of the emissions.

24. A luminescent reagent according to Claim 22, wherein each of said chemiluminescent reactions emits radiation in a different spectral range.

25. A luminescent reagent according to Claim 22

or Claim 23, wherein at least one of said substances is capable of binding or otherwise linking in one of an immunoassay binding reaction, a protein binding reaction a nucleic acid hybridisation reaction and a receptor binding reaction.

26. A luminescent reagent according to any one of Claims 22 to 25, wherein said labelling components are structural variants of the or each other labelling component.

27. A luminescent reagent according to any of Claims 22 to 25, wherein one of the components in at least one of said chemiluminescent reactions is based on acridinium compounds or structural variants thereof.

28. A luminescent reagent according to Claim 27, wherein one of the components in at least one of said chemiluminescent reactions is an acridinium salt alkylated at the ring nitrogen.

29. A luminescent reagent according to Claim 27, wherein one of said components in at least one of said chemiluminescent reactions is an acridinium phenylester wherein the ester is formed at position 9 of the acridine nucleus.

30. A luminescent reagent according to Claim 28, wherein one of said chemiluminescent reactions includes an acridinium phenyl ester wherein the phenyl moiety is substituted with electron withdrawing groups to yield a chemiluminescent reaction in which the emissi n of light occurs ver a relatively short period, and another of said

chemiluminescent reactions includes an acridinium phenyl ester wherein the phenyl moiety is substituted with electron donating groups to yield a chemiluminescent reaction in which the emission of light occurs over a relatively long period.

31. A luminescent reagent according to Claim 27, wherein one of said chemiluminescent reactions includes an acridinium salt which, on triggering of said reaction, emits radiation at a relatively short wavelength and wherein another of said chemiluminescent reactions includes an acridinium salt wherein the electronic conjugation of the acridine nucleus is increased whereby, on triggering of said another reaction, said acridinium salt emits radiation at a relatively long wavelength.

32. A luminescent reagent according to Claim 31, wherein said one chemiluminescent reaction emits radiation of wavelength in the range of from 400 nm to 500 nm and said other chemiluminescent reaction emits radiation in the range of from 500 nm to 700 nm.

33. A luminescent reagent according to Claim 27, or any claim dependent thereon, wherein the acridinium compound is modified or further derivatised to permit covalent coupling to a molecule of biological interest.

34. A luminescent reagent according to any one of Claims 22 to 25, wherein at least one of said chemi-luminescent reactions is based on one of the following compounds:

(i) phthyhaldydrazides and related compounds

(ii) dioxetanes, dioxetanones and related compounds

(iii) Bis-oxalate esters, related compounds and associated acceptor partners where required.

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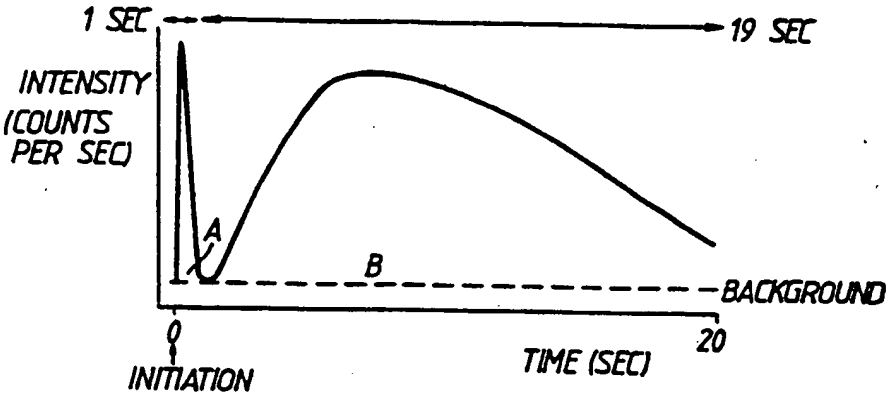
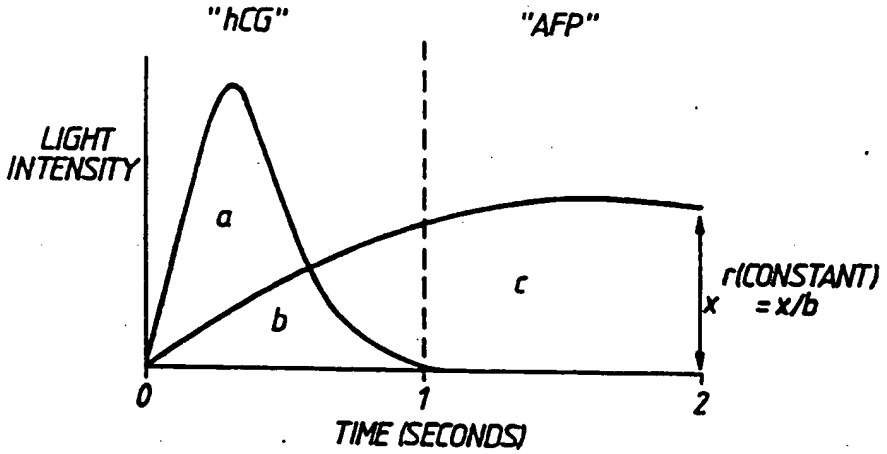


Fig.1.



AFP SIGNAL = c

APPARENT hCG SIGNAL = (a + b)

TRUE hCG SIGNAL = (a + b) - x/r

Fig.2.

INTERNATIONAL SEARCH REP RT

International Application No PCT/GB 90/00957

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: G 01 N 21/76

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
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A	Patent Abstracts of Japan, volume 9, no. 90 (P-350)(1813), 19 April 1985, & JP,A, 59217136 (HITACHI SEISAKUSHO K.K.) 7 December 1984 see abstract ----------	1

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th September 1990	17. 10. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. SOTELO

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		CH-A- 645725	15-10-84
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		FR-A,B 2409516	15-06-79
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		US-A- 4478817	23-10-84

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Published
With international search report.

(54) Title: METHOD AND APPARATUS FOR PERFORMING MULTIPLE SEQUENTIAL REACTIONS ON A MATRIX

(57) Abstract

A method and apparatus are provided for preparing a substrate upon which is located microdrop-sized loci at which chemical compounds are synthesized or diagnostic tests are conducted. The loci are formed by applying microdrops from a dispenser from which a microdrop is pulse fed onto the surface of the substrate.

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METHOD AND APPARATUS FOR PERFORMING MULTIPLE SEQUENTIAL REACTIONS ON A MATRIX

FIELD OF THE INVENTION

The present invention is directed to a method and apparatus for performing sequential reactions on a plurality of sites on a matrix using noncontiguous microdrop-sized loci. The apparatus and method are useful for performing a test or synthesis involving sequential steps such as DNA sequencing, DNA diagnostics, oligonucleotide and peptide synthesis, screening tests for target DNA, RNA or polypeptides, synthesis of diverse molecules, DNA separation technology whereby DNA binds to target molecules, preparation of polysaccharides, methods for making complementary oligonucleotides, and any other test, sequencing or synthetic method utilizing a sequence of steps at a locus. An advantage or improvement can be obtained by providing loci so that combinations of different reactions may be conducted on the same matrix.

BACKGROUND OF THE INVENTION

Methods are known for performing a plurality of sequential tests or reactions at loci on a matrix by attachment of molecules to a solid phase. Typically, a solid phase is prepared having a free functional group such as a hydroxy group, amino group, etc. and linking groups are attached t the surface by way f covalent linkages. These linkers serve as "handles"

to which molecules may be attached for sequential synthesis of such linear molecules as polypetides and polynucleotides. A disadvantage of such solid state synthesis is that the entire substrate or a large portion of the substrate must be exposed to a single reagent, such as the reagent which is the next molecule to be attached to the substrate, a rinsing agent or a deprotecting agent.

In some instances, locations on the substrate can be selectively treated if the reaction to be conducted is photolytic in nature, so masks may be prepared to expose selected areas to the activating radiation. However, an obvious disadvantage is that reactions must be devised which can be conducted by photolytic activation and different masks must be used to shield portions of the substrate at which the reaction is undesired.

The present invention provides a method whereby reactions may be conducted on noncontiguous microdrop-sized loci on a substrate. Since the reagents according to the present invention are in liquid form, virtually any chemical reaction which may be conducted in solution or suspension may be performed.

It is therefore an object of the present invention to provide a method and apparatus for performing a plurality of sequential reactions on a substrate whereby the reactions are conducted on microdrop-sized loci and, if desired, a different sequence of reactions may be conducted at each locus.

Furthermore, an object of the present invention is to provide a method and apparatus for conducting a plurality of sequential reactions on a matrix using

liquid reagents whereby the chemical reactions may be performed in solution or suspension.

These and other objects of the invention will be apparent from the following description, the appended claims and from the practice of the invention as described herein.

SUMMARY OF THE INVENTION

The present invention provides a method and apparatus for performing a plurality of chemical reactions at different sites on a substrate wherein the same or different tests, sequencing or synthetic reactions may be conducted at the loci. The invention provides a substrate having a surface which has chemical moieties that are reactive with reagents that are dispensed from a microdrop dispensing device. These reagents may be molecules that become attached to the surface in the microdrop loci to which they are dispensed, as in the application of activated nucleic acid phosphoramidites, or the reagents may modify the surface in the microdrop loci for subsequent chemical reactions, as in the deprotection of the 5' hydroxyl group during the synthesis of oligonucleotides. In the case of delivery of reagents that become attached to the surface, the invention provides a substrate having a surface to which a first reagent can be attached by dispensing microdrops of the reagent in liquid form onto the substrate. The dispenser is displaced relative to the surface and at least one microdrop is applied thereto containing the same or a different reagent. By repeating this using the same or a different first reagent in liquid form, a plurality of loci on the surface may be prepared wherein the reagents covalently attach at microdrop-sized loci wherein the boundaries of each locus are not contiguous to any adjacent locus. The surface

may then be washed to remove unattached reagent. If needed, the entire surface may be treated, or alternatively, a selected subset of loci may be treated, with deprotecting reagents to expose reactive sites of the molecules attached to the surface. The deprotecting reagent may also be dispensed from the device. Then one or more microdrops containing a second reagent in liquid form may be dispensed at selected loci on the substrate surface, whereby the second reagent is selected to react with the molecules already attached to the matrix. The dispenser is again displaced relative to the surface to apply the second reagent at different loci using the same or a different second reagent which reacts with the respective attached molecules. Again, the entire surface will be washed to remove unreacted second reagents. Then the entire surface or selected subsets of loci may be treated with deprotecting agents, and this process may be repeated.

In the case of delivery of reagents that modify the reactivity of the surface, the invention provides a substrate having a surface to which the reagent is applied by dispensing one or more microdrops onto the substrate. The dispenser is displaced relative to the surface and one or more microdrops are applied thereto. This process may continue until the desired set of microdrop-sized loci have been modified by the application of reagent. The surface may then be washed to remove excess reagent. The entire surface or a selected subset of loci, may be treated with a reagent that becomes attached to the loci modified by the microdrop dispensed reagent, or alternatively a reagent may be applied that becomes attached to the surface except at the loci that were previ usly modified by the microdrop dispensed reagent. If the

reagents that become attached to the surface contain chemical moieties that can be modified by the microdrop dispensed reagent, the process may be repeated such that the same or different loci are modified by the microdrop dispensed reagent and then reacted with a reagent or reagents that become attached to the modified loci until the desired compounds have been synthesized on the substrate.

It will also be recognized that a combination of the above strategies may be employed wherein both the reagents that become attached to the surface in microdrop loci and reagents that modify the surface in microdrop loci are dispensed by the microdrop dispensing device.

Upon completing the desired number of sequential steps at the loci on the substrate, the compounds may be removed selectively or non-selectively, if desired, from the substrate using cleavage reagents which remove compounds bound through linking groups to solid substrates. Cleavage agents include enzymatic or other chemical agents, which may also be dispensed as microdrops at selected loci. It will be appreciated, for example, in the case of diagnostic methods, isolation of the final compound located in each of the loci is not important, therefore cleavage of the compound from the substrate is an optional step.

In some circumstances, it may be desirable to analyze the molecules directly upon cleavage from the substrate by such techniques as mass spectrometry. In such instances, it is desirable to provide a linker (the moiety through which the molecule in question is attached to the substrate) which is cleavable by electron beam, laser, or ther energy

source so that molecules at a locus may be selectively cleaved from the substrate. This is particularly advantageous for analyzing the molecules by mass spectrometry, whereby the laser or electron beam cleaves the molecules from the substrate, ionization occurs, and the ions are accelerated into a mass spectrometer.

The substrate may be a solid, such as glass, prepared to receive linkers attached to the surface. Porous substrates, such as paper or synthetic filters may be used, as well as filters (such as those sold by Nucleopore™) having straight, parallel micropores. In such a microporous substrate, the reactions may take place within the pores, thus amplifying the potential signal at the locus.

It will also be recognized that the present invention provides a method for determining the presence of an analyte in a sample by contacting the sample with a device prepared according to the present invention having a plurality of microdrop-sized loci of covalently attached reagents whereby the analyte binds to at least some of the reagents. Detection of the loci at which binding occurs may be performed by conventional methods such as fluorescence, chemiluminescence, colorimetric detection, radioactive label detection, and the like.

The present invention also provides a method for delivery of microdrops to the substrate that relies on positioning the substrate such that the separation between the dispenser and substrate is less than the separation required for free droplet formation. In this configuration, the liquid column emerging from the nozzle due to the applied pressure pulse impacts upon the substrate before a droplet forms (i.e., a

column of liquid stretches between the nozzle and substrate). The impact upon the substrate alters the flow of liquid from the nozzle such that a much smaller amount of liquid is ultimately delivered to the substrate as compared to the case where distinct droplets are formed. This method allows for much closer spacing of loci on the substrate and higher positional precision for the placement of loci.

DESCRIPTION OF THE DRAWINGS

FIG. 1A shows a substrate having microdrop sized loci on one surface. FIG. 1B shows the cross-section of a microporous substrate with straight, parallel micropores having a microdrop-sized locus containing attached molecules.

FIG. 2 is a schematic side view of a microdrop dispenser and substrate.

FIG. 3 is a schematic illustration of two loci at which different peptides are prepared.

FIG. 4 is a schematic illustration of a flexible, continuous substrate used with the method of the invention.

DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention provides a method for performing a plurality of sequential reactions on a substrate. The surface of the substrate contains chemical moieties that react with reagents that are dispensed from a microdrop dispensing device. The reagents may be molecules that become attached to the surface f the substrate in the microdrop loci to which they are dispensed or th r agents may modify

the surface of the substrate to facilitate the formation of a covalent bond between the surface and a second reagent. In the latter case, the entire surface or a selected subset of loci may then be treated with a second reagent that becomes covalently attached to the loci modified by the first reagent. If only a selected subset of loci are treated with the second reagent, this step may be repeated with a third reagent that becomes attached to another subset of loci modified by the first reagent.

The present invention may be utilized to prepare, for example, molecules such as peptides. In a preferred embodiment a linker molecule is provide as the first reagent whereby one end of the linker will be attached to the substrate surface. The other end of the linker will be adapted to form a linkage with the carboxy terminal of an amino acid or peptide, to form, for example, an amide or ester linkage. This end of the linker may be initially chemically protected by protecting groups such as t-butoxycarbonyl groups (t-BOC) or other protecting groups known in the peptide synthesis art. By application of a second reagent onto the locus which removes a protecting group, such as acid solution, the protecting group may be removed. The next reagent applied at each locus would then be an amino terminal-protected and side-chain protected amino acid or polypeptide, preferably having an activated C-terminal group for linking the C-terminal to the end of the linker. This process may be repeated with the same or different amino acids or peptides at each of the microdrop loci until the substrate includes the peptides of desired sequences and lengths. Thereafter, the protective groups are removed from some or all of the peptides, as desired. The deprotection may be achieved using a common deprotection agent, which removes the protecting

groups on side chains and the amino ends simultaneously, as is known in the peptide synthesis art. The peptides may be cleaved from the linker using methods known to those of ordinary skill in the peptide synthesis art which cleave peptides from a solid support as, for example, used in the Merrifield synthesis technique.

It will be realized that a particular advantage of this method is that, by keeping a record of the reagents utilized at each of the microdrop sized loci, peptides of different lengths and sequences maybe made concurrently on the same substrate. Such peptides may have a variety of uses including, but not limited to, screening for biological activity whereby the respective peptide sequences at each locus is exposed to a labeled or unlabeled peptide receptor, such as an antibody, a cell receptor, or any other variety of receptor.

The method according to the present invention may also be utilized to prepare oligonucleotides by sequentially dispensing through the microdrop dispenser protected nucleic acids. These may be added sequentially at each locus using the same or different nucleic acids or polynucleotides. Preferably, the 3'-end of the oligonucleotide will be attached to the linker molecule and the oligonucleotide will be synthesized from the 3' end to the 5' end using known techniques for oligonucleotide synthesis. The protecting groups are preferably those known in the oligonucleotide synthesis art. The oligonucleotide may be utilized, for example, for hybridization with an unknown oligonucleotide to determine the sequence of the unknown oligonucleotide.

An oligonucleotide synthesized at one locus may be

utilized to synthesize its complementary oligonucleotide by using DNA polymerase. Preferably, the locus will comprise straight pores in a porous substrate. The complementary oligonucleotide may then be removed by washing a denaturing agent through the pores onto a new substrate, thereby resulting in one substrate (the original porous substrate) containing the oligonucleotides which were originally synthesized, and another substrate containing their complements.

An array of synthesized oligonucleotides may be used to generate an array of complementary oligonucleotides by using pre-synthesized oligonucleotides, optionally containing a reactive chemical moiety such as a spacer with a primary amine that attaches to the phosphate chain. In this embodiment, the pre-synthesized oligonucleotides are hybridized to the array of oligonucleotide prepared with the microdrop dispenser. The localized complementary oligonucleotides are preferably removed from the synthesized array in denaturing conditions and washed onto a second substrate. This second substrate is preferably a material such as a nylon or nitrocellulose membrane, or surface with amino reactive linkers, where the oligonucleotides become immobilized. Preferably a flow system onto the second substrate is utilized such that the net flow is essentially perpendicular to the original substrate so that the complementary oligonucleotides in adjacent loci do not intermingle. This may also be accomplished by employing an electric field that is perpendicular to the original substrate such that the complementary oligonucleotides electrophorese onto the second substrate.

In yet another embodiment of the present invention the substrate to which oligonucleotides are attached may

be used as a tool in gene therapy whereby mutations may be identified in a genome. For example, oligos complementary to fragments of the known sequence of the normal gene may be attached to the substrate. Digestion of a single strand of the gene from the subject in question and contact with the substrate containing complementary oligo sequences may reveal oligos to which there is binding, thereby indicating the presence or absence of fragments in the subject's genome.

The substrate containing oligos may also be used to identify DNA in samples from the environment to detect, for example, the presence or absence of certain species, in the case where the DNA sequences are known, or to determine the presence of DNA fragments which anneal to the substrate in the case where the DNA sequences are unknown. The oligonucleotides may thereafter be amplified by PCR amplification technology.

If the substrate is a porous filter, membrane or other material which can be cut, the substrate may be divided into portions containing one locus (or a plurality of loci having identical or different molecules). These portions may be placed in microtiter wells for diagnostic or therapeutic tests whereby each well is separately treated with a sample.

One application of the present invention is to prepare an array of oligonucleotides for the sequencing of DNA by hybridization. The basis for this method is that a given sequence can be constructed from the knowledge of its constitutive set of overlapping sequence segments, provided there is a certain degree of uniqueness among these segments. The set of overlapping sequence segments of length *n* can be

obtained by hybridization of the unknown DNA to a set of *n*-mer oligonucleotides which represent all 4^n possible sequences. The advantages of sequencing by hybridization include faster sequence determination, lower cost, ease of automation and higher reliability (as compared to a single sequence reading from a gel). For an array of oligonucleotides of length *n* it is possible to determine the average length of DNA fragment that can be unambiguously sequenced. Although difficulties can arise when a fragment of length *n-1* appears in the sequence more than once, nevertheless, statistical analyses have shown sequencing by hybridization to be a feasible method. The relationship between the length of oligonucleotides and the length of the average resolvable sequence has been determined. Typical numbers are shown in Table 1. For example, an array of all 65,536 octamers can be used in the sequencing of short, 100 to 200 base pair fragments.

Furthermore, it has been shown that inclusion of a random content fixed length gap in the oligonucleotides of the array can be used to achieve higher lengths of sequence resolution. The combination of an array of all 4^8 octamers and an array of all 4^8 octamers with a random nucleotide inserted in the middle of the octamer has nearly the same resolving power as an array of all 4^9 nonamers, even though the nonamer array is twice as large.

The preferred ink jet device utilized to deliver the microdrops generates addresses less than 100 microns across, and address sizes as small as 10 microns are attainable. A primary advantage to use of the ink jet is that standard methods for oligonucleotide

synthesis that have been optimized for extremely high yields can be employed.

By employing a multiple jet device the synthesis of complete arrays of oligonucleotides can proceed four times faster and with less material than can be accomplished by performing only addressable deprotection. The simplest design to accomplish this is a five jet system, one jet each for the four phosphoramidite reagents and one jet for the activating tetrazole solution. The operation of this device is directly analogous to the operation of color ink jet printers. In every coupling cycle, for each address on the array a number is assigned to indicate the correct synthon to be added. During the reagent delivery process, the stage rasters through the addresses of the array. Tetrazole is first applied to the substrate. At each address an additional offset motion is applied to bring the correct phosphoramidite jet (A, C, G or T) in line. One or more droplets of the phosphoramidite are then dispersed. Subsequent to this a second offset motion is employed to bring the tetrazole jet in line with the address. After dispersal of the tetrazole reagent, the stage can raster to the next address for a new delivery cycle. The software for the advanced device is very similar to the control software described in the examples with a modification that a 'color' bitmap is used to represent the array. The four phosphoramidite reagents are each assigned to a specific color. During the raster through the array for delivery, the color at each pixel in the bitmap is translated to the offset motion to bring the correct reagent in line with the address. The tetrazole jet fires at every address position.

The tetraethylene glycol linker is useful for single hybridization with oligonucleotides. Low non-specific binding has been observed. Longer polymers of ethylene glycol may be utilized, as well as modified phosphodiesters. Phosphoramidite reagents are commercially available that may be polymerized in a stepwise manner to yield dimethoxytrityl-capped linkers of virtually any length desired. Since this linkage is ultimately a phosphodiester with phosphates spaced by alkyl chains of only a few carbons, it will have similar hydrophilicity to standard DNA. Further, since the linker is negatively charged at neutral pH, lower non-specific binding of DNA to the substrate is expected.

To address the question of coupling efficiency and thus the sequence fidelity in the synthesized arrays the preferred method is to synthesize large arrays, where all addresses contain the same sequence, and perform Maxam-Gilbert sequencing directly on the substrate region that contains the array. Prior to the start of sequencing the array may be end-labeled with ^{32}P phosphate.

The sequencing by hybridization may require either larger arrays, for example the undecamer array, or arrays that have been optimized to obtain more information from a set of hybridization tests. For such large arrays, the complete set of undecamers has 4.2 million members, therefore small address and guard regions are advantageous. With 100 micron addresses and 50 micron guard regions, parameters that are within the capacity of examples disclosed herein, the entire undecamer array would occupy an area of 10.5x10.5 square inches.

In yet another embodiment the microdrops may be used to synthesize polysaccharides using the monosaccharides as building blocks. However, it will be readily apparent that many other types of polymeric materials may be made according to the present invention whereby the same or different polymers maybe be constructed at each locus on the substrate.

In a particularly advantageous use of the present invention, small molecules may be made whereby the molecules may be built sequentially using reagents in a multistep synthesis. These need not be polymeric molecules where there is a repetitive unit. Since different reagents may be applied to one or more of the loci on the substrate, there is an advantageous diversity of structures that can be attained by the multiple and concurrent synthesis technology according to the present invention. The target compounds may be contemporaneously, but separately synthesized on the substrate to generate an ensemble of compounds which may or may not be structurally related. Each step of the synthesis which occurs at each locus should involve soluble reagents, and should occur at a reasonable yield at typical ambient temperatures, since most or all of the sites on the substrate will be essentially isothermal. For example, a benzodiazepine may be prepared from an amino acid bound to the substrate by the carbon terminus. Treatment with a microdrop containing 2-aminobenzophenone imine forms a substrate-bound imine and then treatment with TFA (trifluoro acetic acid) generates a benzodiazepine. By using different amino acids and different aminobenzophenones, an array of different benzodiazepines maybe made in this manner.

Reference will be made now to the various figures which further describe the preferred modes for practicing the invention.

Referring now to the figures, in Fig. 1A there is shown a substrate 20A having on one surface thereof the microdrops 21 which define each locus at which the chemical synthesis or diagnostic reaction may take place according to the present invention. Since each microdrop is discrete and noncontiguous with adjacent microdrops, reactions may be conducted at each microdrop which are independent of reactions at other microdrops. In Fig. 1B, there is shown a microporous substrate 20B having straight, parallel micropores 40. The growing chains of molecules (41) may be attached within the pores, thus amplifying the synthesis by the additional surface area available beneath the surface of the substrate.

Referring to Fig. 2 there is shown a schematic elevation of the substrate 20 upon which is located on one surface thereof the microdrops 21. Schematically shown is the microdrop multiple jet head dispenser 22 from which, as shown, is being dispensed a microdrop 23. The microdrop is dispensed by a pressure pulse generating means 24, such as a piezoceramic driven pressure pulse device as is typically known in the art of inkjet printers. The timing and amplitude of the pulse are controlled by a suitable electrical controller 25. The location of the dispenser 22 may be suitably controlled by a computer controlled mechanical grid or arm by which precise movements of the dispenser to different locations over the surface 20 can be controlled by control means 26. A reagent source 28 may serve as a reservoir for a particular reagent which is being dispensed, with the flow of the reagent being

controlled by a flow controller 27. Alternatively, the dispenser 22 may be held stationary and the substrate 20 may be moved by appropriate controllers in a precise way to locate the microdrops on the substrate surface 20. As part of the control of the location of the dispenser 22, the controlling means 26 will also contain a memory to record the identity of each reagent and the sequence at which they were added to each microdrop locus.

Referring to Fig. 3, there is shown an elevational view of the substrate 20 and a schematic view of the elements which may be present at two of the microdrop loci. At each of the loci there is a plurality of chemical linkers 30 which are attached at one end to the substrate surface 20 and at the other end to a molecule which is being synthesized at the particular locus. In the figure the letter "A" represents an amino acid. By separate microdrop treatments in one locus the peptide having the sequence (using conventional peptide nomenclature whereby the last amino acid added to the chain is the N-terminus) the peptide $A_3A_2A_1$ has been made by applying in sequence the reagents containing the amino acids A_1, A_2 and A_3. At the other microdrop location the peptide $A_3A_4A_1$ has been made by applying in sequence the amino acid reagents containing A_1, A_4 and A_3.

Referring to Fig. 4, there is shown a schematic diagram of one embodiment of an apparatus utilizing the present invention. The substrate 35 is a continuous, flexible material to which chemicals may covalently linked, such as flexible polystyrene having surface groups to which chemical linkers may be attached, such as those used in solid phase peptide synthesis. One or more el ctromechanically controlled dispensers 36A and 36B are used to apply

microdrops onto the substrate 35. The movement of the substrate 35 is also electromechanically controlled in the longitudinal direction shown by the arrow. The movement of the dispensers 36A and 36B may be controlled along the transverse direction, as well as along the longitudinal direction. Excess reagent is washed off in a bath in tank 37. Detecting means 38, which is also controlled in the transverse and lateral directions, is utilized to observe the loci for either quality control or, in the case of a diagnostic use, for a signal such as fluorescence, radioactivity, polarization, chemiluminescence, etc.

EXAMPLE 1

INK JET DEVICE

A device for reagent delivery was constructed consisting of two 25 mm micrometers that provide *x* and *y* translation coupled to 10 V, 0.5 amp per phase, 200 step per revolution stepper motors. A single motor step gives a travel of 2.5 μm. A 48-V power supply with dropping resistors was incorporated to increase high speed motor torque. A piezoelectric ink jet head was mounted vertically to a third 12.5 mm micrometer. The jet was positioned to fire droplets upwards to the underside of a microscope slide held to the top of a platform with a spring loaded slide holder. Electric pulses were generated with electronics that allow all pulse parameters, such as the driving voltage, pulse duration and frequency, to be adjusted. A video camera, which translates in *x* and *y* with the jet, was positioned above the slide to monitor drop ejection by focusing on the lower slide surface. Alternately, the camera could be rotated to view across the jet nozzle with

lighting provided by a strobed LED to allow for visualization of ejected droplets.

The ink-jet device was controlled by C/C++ program ASyn, with a Windows interface incorporated such that nearly all functions can be done with a mouse which can be placed inside a glove box along with the ink jet device. ASyn provides TTL level triggering to peripheral hardware through a multi-parallel port add-in card on a PC compatible computer.

The software allows for several modes of operation including a manual move and fire, a drawing mode that 'prints' a bitmap image, and a macro execution mode that can 'print' a number of images at different locations. A bitmap is a numerical representation of a two dimensional image made up of an array of pixels. In the case of black and white image, a 1 in the bitmap produces one color while a 0 produces the other. Thus the four bytes FF, 0, FF, and 0, whose binary 'bitwise' representation is 11111111, 00000000, 11111111, and 00000000 would produce alternating white and black lines 8 pixels wide if rendered on a computer screen as a bitmap. The logic of the program divides the arrays into 'addresses' and 'guard' positions that can have variable dimensions. The decision to fire at a given address is determined by the value of a pixel in the bitmap image. The mode of firing at an address can also be controlled to give single or multiple droplets in the center of the address as well as a pattern of single droplets to fill a square address area. In addition, logic has been incorporated into ASyn to generate the appropriate bitmaps for the synthesis of combinatorial arrays of oligonucleotides.

A variety of organic solvents including dibromomethane, nitromethane, acetonitrile and dimethyl formamide were found to be suitable for ink jet delivery. Dichloromethane was not found to be suitable for room temperature delivery although a cooled jet assembly provided better results. A reagent consisting of 0.8 M $ZnBr_2$ in 9:1 nitromethane:isopropanol has been selected for the deprotection of the 5'O dimethoxytrityl protected deoxyribose during the on-chip synthesis of oligonucleotides.

While delivering water, the ink-jet pulse parameters can be readily adjusted for the delivery of single droplets free of satellites. When the jet nozzle to microscope slide separation is greater than 100 microns, the drop footprint on a glycidoxypropyl silanized slide can be varied from ~150 to ~250 microns depending on the driving voltage. When the nozzle to slide separation is less than 60 microns, the footprint is seen to decrease to between 60 and 80 microns. In this case the footprint is relatively independent of the driving voltage.

The driving pulse for the ink jet is optimized by setting the video camera to view across the nozzle of the jet with the LED strobe in the background. Driving voltage and delay parameters are adjusted while firing a continuous stream of droplets. It was found that the deprotecting reagent required a driving voltage that was approximately one third that which was required for water. A high degree of control can be exerted on the droplet size when firing deprotection reagent by adjusting the driving voltage. In the case of firing single droplets onto a slide, the size of the 'fo tprint' of the droplet as it spread onto the slide surface could be varied

from less than 100 μm to more than 250 μm by varying the drive voltage. A combination of suitable driving voltage in close positioning has yielded the delivery of droplets of deprotecting agent with a footprint on the order of 60 microns.

EXAMPLE 2

OLIGONUCLEOTIDE SYNTHESIS

Oligonucleotide synthesis was performed using the ink jet to deliver deprotecting reagent.

A standard microscope slide was coated with glycidoxypropyl silane and reacted with tetraethylene glycol. A standard phosphoramidite synthetic cycle was used. The entire synthesis was performed in a dry nitrogen filled glove box. Prior to the first coupling reaction the slide was rinsed with acetonitrile (MeCN, distilled from calcium hydride) and dichoromethane (DCM) and vacuum dried for one minute. Phosphoramidite monomers were dissolved a 0.1M in acetonitrile. Tetrazole was dissolved at 0.5M in MeCN. Coupling was performed by adding 80 μl each of the tetrazole and phosphoramidite to an aluminum reaction trough. The glass slide was placed into the trough causing the liquid to spread evenly over the slide surface. Reaction was allowed to proceed for three minutes. The slide was then rinsed with MeCN and the coupling procedure repeated.

After coupling, the slide was dipped for two minutes into a Teflon and glass chamber that contained an oxidizing iodine/lutidine/MeCN/water solution purchased from Pharmacia (250μl each of Oxidation 1 and Oxidation 2). The slide was then rinsed twice with MeCN and DCM and dried in vacuum.

After drying, the slide was placed onto the ink jet platform for spraying of the appropriate pattern of deprotection reagent. The slide was allowed to sit for a period of five minutes after that last droplet was delivered. The slide was then rinsed twice with MeCN and DCM and vacuum dried in preparation for the next coupling cycle.

A the end of synthesis the slide was removed from the glove box and immersed overnight in a bath of 30% ammonia at room temperature.

A test of simple oligonucleotide synthesis was performed to generate 4x5 arrays of poly-T. In this study, 17 cycles of coupling were performed using a single spray pattern that deposited 15 droplets to all addresses. The addresses were spaced on 2 mm centers. At the end of synthesis the oligonucleotides were deblocked and hybridized with an end-labeled 15-mer of poly-A using 6x SSC/0.5%SDS and 400 ng of end-labeled probe. The synthesis of arrays of poly-T was successful.

It will be appreciated that the above described is intended to be illustrative and not restrictive and that many embodiments will be apparent to those with skill in the art upon reviewing the above description and following claims. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims along with the full scope of equivalents to which such claims are entitled.

Table 1. Length of possible sequence determination versus the length of oligonucleotides used for hybridization.

Length of Oligonucleotide	Length of Sequence Identifiable*
7	80
8	180
9	260
10	560
11	1300
12	2450

*These numbers represent the length for which sequence reconstruction will be possible in 95% of all cases.

CLAIMS:

1. A method of step-by-step synthesis of an array of different chemical compounds at microdrop-sized loci, where each compound is covalently attached to or beneath the surface of a substrate comprising the steps of:

(a) applying through a single unit of a multiple reagent dispenser at least one microdrop of a first reagent in liquid form to said surface, wherein said substrate is chemically prepared to react with said first reagent to covalently attach said reagent to said substrate;

(b) displacing said multiple reagent dispenser relative to said surface, or the surface with respect to multiple reagent dispenser, and applying at least one microdrop containing either the first reagent or a second reagent from a different dispenser unit to said surface wherein said substrate is chemically prepared to react with said reagent to covalently attach said reagent to said substrate;

(c) optionally repeating step (b) at least one time using the same or different reagents in liquid form from different dispenser units wherein each of said reagents covalently attaches to said substrate to form covalently attached compounds;

(d) washing said substrate to remove unattached reagents;

(e) modifying said attached reagents;

(f) repeating steps (a) through (e) with the same or different reagents at various loci on the substrate; and

(g) optionally, selectively or nonselectively removing the attached compounds at said loci from said substrate.

2. A method of step-by-step synthesis of an array of different chemical compounds at microdrop-

sized loci, where each compound is covalently attached to or beneath the surface of a substrate comprising the steps of:

(a) applying through a single unit of a multiple reagent dispenser at least one microdrop of a first reagent in liquid form to said surface at the first locus;

(b) displacing the multiple reagent dispenser relative to said surface and applying through a second unit of a multiple reagent dispenser a second reagent to the first locus to form a mixture of reagents at the first locus wherein said substrate is chemically prepared to react with said mixture to covalently attach one or more of said reagents to said substrate at the first locus;

(c) optionally repeating steps (a) and (b) at additional microdrop-sized loci of said surface where the same or different mixture of reagents are formed at each locus.

(d) washing said substrate to remove the excess reagents;

(e) modifying said attached reagents;

(f) repeating steps (a) through (e) with the same or different reagents at various loci on the substrate; and

(g) optionally, selectively or nonselectively removing the attached compounds at said loci from said substrate.

3. A method according to claim 1 or 2 wherein said substrate comprises a solid, non-porous material.

4. A method according to claim 1 or 2 wherein said substrate comprises a porous material.

5. A method according to claim 4 wherein

said porous material comprises paper.

6. A method according to claim 4 wherein said porous material comprises a sheet having internal straight, essentially parallel pores.

7. A method of preparing a substrate according to claim 1 or 2 wherein said reagents in said microdrops comprise protected or unprotected amino acids, thereby forming polypeptides attached at said loci.

8. A method of preparing a substrate according to claim 1 or 2 wherein said reagents in said microdrops comprise protected or unprotected nucleic acids, thereby forming oligonucleotides attached at said loci.

9. A method of preparing a substrate according to claim 1 or 2 wherein said reagents in said microdrops comprise protected or unprotected sugars, thereby forming oligosaccharides attached at said loci.

10. A method according to claim 8 further comprising the step of amplifying said oligonucleotides by polymerase chain reaction amplification.

11. A method according claim 8 further comprising the steps of forming the oligonucleotide complements of said attached oligonucleotides by use of polymerase and nucleic acids, and washing said complements from said substrate with a denaturing agent.

12. A method according to claim 8 further comprising the steps of hybridizing oligonucleotide complements to said attached oligonucleotides, and

washing said complements from said substrate with a denaturing agent.

13. A method according to claim 12 wherein said substrate comprises a porous membrane having straight, essentially parallel pores and said complements are removed through said pores onto a second substrate, wherein the relative locations of said complements on said second substrate correspond to the relative locations of their respective oligonucleotides on said substrate from which they were removed.

14. A method according to claim 1 or 2 wherein said reagents in said microdrops comprise chemical moieties for stepwise synthesis of target compounds, thereby forming said target compounds attached at said loci.

15. A method according to claim 1 or 2 wherein said compounds attached at said loci are cleaveable from said substrate by exposure to laser or electron beams.

16. A method according to claim 1 or 2 wherein said compounds attached at said loci are cleaveable from said substrate by chemical reagents.

17. A substrate prepared according to the method of claim 1 or 2.

18. A substrate prepared according to the method of claim 7.

19. A substrate prepared according to the method of claim 8.

20. A substrate prepared according to the method of claim 9.

21. A substrate prepared according to the method of claim 13.

22. A method for determining the presence of an analyte in a sample comprising the steps of contacting said sample with a substrate having a surface on which are contained microdrop-sized loci of covalently attached reagents, whereby said analyte binds to at least some of said reagents at said loci; and detecting the loci at which said binding occurs.

23. A method for detecting active molecules in a sample comprising the steps of contacting said sample with a substrate having a surface on which are contained microdrop-sized loci of covalently attached reagents; whereby said active molecules bind to at least some of said reagents at said loci; and detecting the loci at which said binding occurs.

24. A method according to claim 22 or 23 wherein said substrate is divided into separate portions and said portions are separately contacted with a sample.

25. A method according to claim 1 or 2 wherein said multiple reagent dispenser consists of multiple piezoelectric jet heads.

26. A method according to claim 25 wherein said quantities of first or subsequent reagents dispensed are microdrops having volumes of from 10 to about 150 picoliters.

27. A method according to claim 1 r 2

wherein said quantities of first or subsequent reagents dispensed are microdrops having volumes of from about 10 to about 150 picoliters.

28. A method of step-by-step synthesis of an array of oligonucleotides at microdrop-sized loci where each oligonucleotide is covalently attached to or beneath the surface of the substrate comprising the steps of:

(a) applying through a single unit of a multiple reagent dispenser at least one microdrop of a solution of tetrazole to the first locus;

(b) displacing said multiple reagent dispenser relative to said surface and applying through a different unit of a multiple reagent dispenser at least one microdrop of a solution of a nucleotide phosphoramidite to the first locus where the phosphoramidite and tetrazole reagents react with said surface to covalently attach the nucleotide to the surface through a phosphite bond;

(c) optionally repeating steps (a) and (b) at additional microdrop-sized loci of said surface where in step (b) the same or different nucleotide phosphoramidite is applied and the nucleotide becomes covalently attached to the surface;

(d) washing said substrate to remove unattached reagents;

(e) applying an oxidizing reagent to the entire substrate to modify the newly formed covalent attachments to phosphodiester bonds;

(f) washing said substrate to remove oxidizing agents;

(g) applying a deprotecting reagent to remove protecting groups to allow for attachment of additional nucleotides;

(h) washing said substrate to remove deprotecting reagent;

(i) optionally repeating steps (a) through (h) where the same or different nucleotide phosphoramidites are applied to the surface loci;

(j) optionally removing protecting groups from the oligonucleotides; and

(k) optionally selectively or nonselectively removing the attached oligonucleotides at said loci from said substrate.

29. A method of preparing a substrate having a plurality of non-contiguous microdrop-sized loci on a surface of said substrate, wherein at each of said loci a compound is covalently attached to or beneath said surface, comprising the steps of:

(a) applying through a single reagent dispenser to a first locus of said surface a quantity of a first liquid activating reagent wherein said reagent modifies said surface at said first locus to activate said surface for forming covalent bonds;

(b) displacing said single reagent dispenser relative to said surface and applying a quantity of said first activating reagent to said surface at a second locus and optionally repeating the displacing and applying steps at third and subsequent loci, each such locus thereby becoming modified locus;

(c) washing the substrate to remove excess reagent; and

(d) applying a quantity of a second reagent to the entire substrate, including loci modified by a steps a, b and c to yield the second reagent covalently bound to said surface at said modified loci.

30. The method of claim 29 wherein said first activating reagent is a deprotection reagent which removes protecting groups of said locus.

31. The method of claim 29 wherein said first activating reagent is an activation reagent which activates reactive groups of said locus.

32. The method of claim 29 wherein step (d) is repeated with the same or a different second reagent where the same or different loci have been modified in accordance with steps (a) through (c).

33. The method of claim 32 wherein said second reagent comprises protected or unprotected amino acids, thereby forming polypeptides attached at said loci.

34. The method of claim 32 wherein said second reagent comprises protected or unprotected nucleotides, thereby forming oligonucleotides attached at said loci.

35. The method of claim 34 wherein said activating reagent is selected from the group consisting of trichloroacetic acid in dichloromethane and zinc bromide in nitromethane:isopropanol.

36. The method of any of claims 29 through 34 wherein said quantity of activating reagent is applied by a pulse from a single piezoelectric jet head.

37. The method of claim 36 wherein said quantity of activating reagent dispensed is a microdrop having a volume of from about 10 to about 150 picoliters.

38. The method of claim 29 wherein said quantity of activating reagent dispensed is a microdrop having a volume of from about 10 to about 150

picoliters.

39. The method of any of claims 29 through 34 wherein said substrate comprises a solid, non-porous material.

40. The method of any of claims 29 through 34 wherein said substrate comprises a porous material.

41. A substrate prepared according to the method of any of claims 29 to 31.

42. The substrate according to claim 41 wherein said substrate comprises a solid, non-porous material.

43. The substrate according to claim 41 wherein said substrate comprises a porous material.

44. A substrate prepared according to the method of claim 32 wherein repeated application of the same or different second reagent results in the formation of polymers covalently bound to said surface at said loci.

45. The substrate according to claim 44 wherein said polymer is a peptide.

46. The substrate according to claim 44 wherein said polymer in an oligonucleotide.

47. A method of preparing a substrate having a plurality of non-contiguous microdrop-sized loci on a surface of said substrate, wherein at each of said loci a compound is covalently attached to or beneath said surface, comprising the steps of:

(a) positioning a piez electric jet

head dispenser at a predetermined distance from said substrate and applying at least one microdrop of a liquid reagent;

(b) displacing said dispenser relative to said surface and applying at least one microdrop of said liquid reagent to said surface at a second, non-contiguous locus, and optionally repeating the displacing and applying steps at third and subsequent non-contiguous loci; and

(c) wherein said predetermined distance is less than the separation distance required for free droplet formation, whereby a smaller volume of liquid reagent is delivered to said substrate per microdrop as compared to the volume per microdrop when a droplet is allowed to form before contact with said substrate.

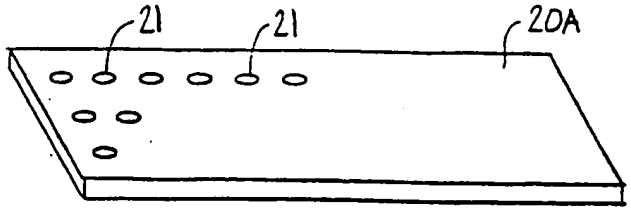

FIG. 1A.

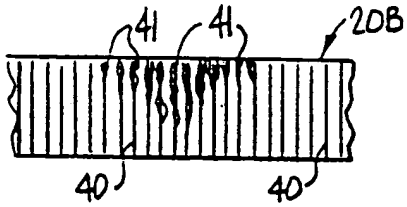

FIG. 1B.

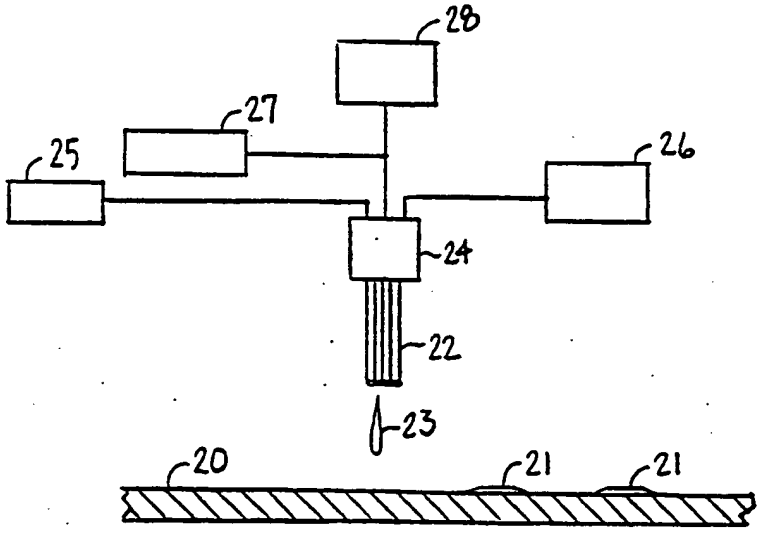

FIG. 2.

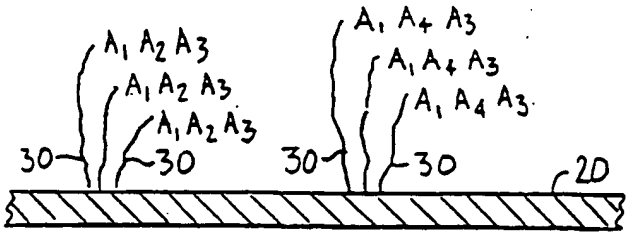
A1 A2 A3
A1 A2 A3
A1 A2 A3
A1 A4 A3
A1 A4 A3
A1 A4 A3
30
30
30
30
20

FIG. 3.

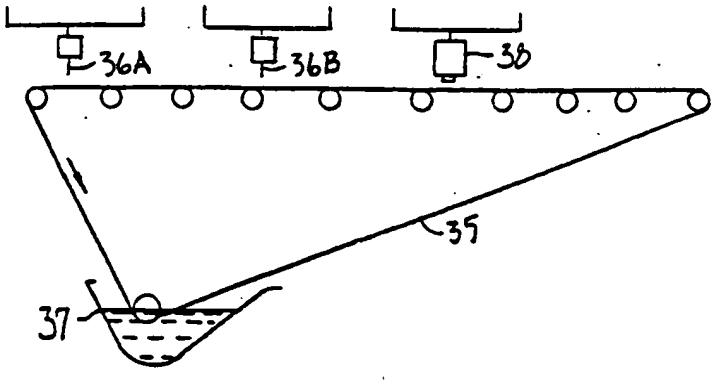
36A
36B
36
35
37

FIG. 4.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03143

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07H 21/00; C12Q 1/68
US CL :435/6; 536/25.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 91.1, 91.2, 290, 810; 436/501; 536/22.1, 23.1, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/00626 (BEATTIE) 25 JANUARY 1990, SEE ENTIRE DISCLOSURE.	1-47
Y	US, A, 5,143,854 (PIRRUNG ET AL.) 01 SEPTEMBER 1992, SEE ENTIRE DISCLOSURE.	1-47
A	WO, A, 92/10588 (FODOR ET AL.) 25 JUNE 1992, SEE ENTIRE DISCLOSURE.	1-47
Y	WO, A, 93/17126, (CHETVERIN ET AL.) 02 SEPTEMBER 1993, SEE ENTIRE DISCLOSURE.	1-47
Y	WO, A, 89/10977 (SOUTHERN) 16 NOVEMBER 1989, SEE ENTIRE DISCLOSURE.	1-47
Y	WO, A, 90/03382 (SOUTHERN ET AL.) 05 APRIL 1990, SEE ENTIRE DISCLOSURE.	1-47

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 MAY 1995	Date of mailing of the international search report 08 JUN1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ARDIN MARSCHEL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03143

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOMOLECULAR STRUCTURE & DYNAMICS, VOLUME 11, NUMBER 3, ISSUED 1993, LIPSHUTZ, "LIKELIHOOD DNA SEQUENCING BY HYBRIDIZATION", PAGES 637-653, SEE ENTIRE DISCLOSURE.	1-47
X --- Y	GENOMICS, VOLUME 13, ISSUED 1992, SOUTHERN ET AL., "ANALYZING AND COMPARING NUCLEIC ACID SEQUENCES BY HYBRIDIZATION TO ARRAYS OF OLIGONUCLEOTIDES: EVALUATION USING EXPERIMENTAL MODELS", PAGES 1008-1017, SEE THE ENTIRE DISCLOSURE.	1-6, 8, 12-17, 19, 21-24, 28-32, 34, 39-44, 46 --- 7, 9, 10, 11, 18, 20, 25-27, 33, 35-38, 45, 47

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03143

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, MEDLINE, WPI, BIOTECH ABS, CAS
search terms: array, insitu, synthesis, hybridiz?, microdrop, microchip